ヒト妊娠子宮内組織におけるエンドセリン濃度およびエンドセリン受容体発現に関する研究(Dissertation_全文)

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Endothelin Receptors in the Human Amnion, Chorion Laeve, Decidua Vera and Placenta

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Endothelin-1-Like Immunoreactivity and Endothelin Receptors in the Human Placenta from Normotensive and Hypertensive Pregnancies

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Running title: Endothelin and its receptors in human placenta

Abstract

The levels of endothelin-1-like immunoreactivity (ET-1-LI) and characteristics of endothelin receptors in the chorionic villous tissue of human placenta were determined. The ET-1-LI level in chorionic villous tissue obtained from normal term placenta was 2,450 ± 940 pg/g wet weight (mean ± SD, n = 4). Further analysis using gel permeation chromatography and reverse-phase high performance liquid chromatography showed that the main ET-1-LI constituent of this tissue was ET-1. Scatchard analysis of [^{125}I]ET-1 binding to the membrane fraction of chorionic villous tissue obtained from term placenta showed high affinity receptor sites with an apparent dissociation constant (Kd) of 23.6 ± 11.1 pM and Bmax value of 388 ± 238 fmol/mg protein (n=5). The same binding study with [^{125}I]ET-3 showed a Kd of 13.9 ± 3.8 pM and a Bmax value of 176 ± 78 fmol/mg protein (n=5). These results suggest that both ET-A and ET-B receptors (ET-AR and ET-BR) are expressed in chorionic villous tissue. This finding was further confirmed by Northern blot analysis showing the expression of both ET-AR and ET-BR mRNAs in this tissue.

ET-1-LI in the umbilical venous plasma of the newborns from women with pregnancy-induced hypertension (PIH) (38.3 ± 10.4 pg/mL, n=5) was significantly ($P < 0.05$) higher than that in the normal newborns from normotensive pregnant women (26.3 ± 5.2 pg/mL, n=12). However, in placental chorionic villous tissue obtained from PIH women, both ET-1-LI level and ET binding profile were not different from those in chorionic villous tissue from normotensive pregnant women.

These results suggest that the abundant ET-ET receptor system is present in the placental chorionic villous tissue and that this system is not the major factor of the pathogenesis of placental dysfunction occurring in

PIH because these systems are similar in normotensive and hypertensive pregnancies.

Key words: endothelin, endothelin receptor, Northern blotting, placenta, pregnancy-induced hypertension.

Introduction

Endothelin (ET) is a potent vasoconstrictor peptide originally isolated from the culture medium of porcine aortic endothelial cells [36]. Three distinct ET-related genes have been identified from a human genomic DNA library and the corresponding peptides were designated as ET-1, ET-2 and ET-3 [12]. To date, ET-1 has been found to be secreted by a wide variety of cells including human breast epithelial cells [2], human macrophages [6], human amnion cells [8, 31], and human placenta [12, 22]. ET has been postulated to be involved in the pathophysiological mechanisms of essential hypertension [25], cerebral vasospasm after subarachnoid hemorrhage [28], renal failure [15] and pulmonary hypertension [36]. In pregnant women with pregnancy-induced hypertension (PIH), plasma ET level has been reported to be increased compared to that in normotensive pregnant women [5, 11, 13, 17, 20, 28, 32].

Recently, cDNAs encoding two distinct subtypes of ET receptor were cloned and termed ET-AR (ET-1-selective type) and ET-BR (non-isopeptide-selective type) [1, 10, 21, 36]. ET-1 binding sites were reported to be present in the feto-placental vessels of normal pregnancy [9, 23]. In pregnancy complicated with intrauterine growth retardation (IUGR), ET-1 binding characteristics of feto-placental vessels were not different from those in normal pregnancy [18]. However, the involvement of ET and ET receptors in chorionic villous tissue in the placental dysfunction of PIH has not yet been determined. In the present study, to elucidate the possible role of placental endothelin in the pathophysiology of PIH, the concentration of ET and its receptors in the placental chorionic villous tissue, as well as ET concentration in the umbilical venous plasma of the newborns, were determined in normal and PIH pregnancy.

Materials and Methods

Subjects: Placentae were obtained from four pregnant women with PIH who had undergone Cesarean section due to fetal distress (mean ± SD of gestational age; 36.2 ± 4.3 weeks), and five normal pregnant women who had undergone elective repeat Cesarean section (gestational age; 38.7 ± 1.1 weeks). Fetal distress was diagnosed by abnormal findings in FHR-monitoring and pulsed Doppler blood flow velocity waveform analysis of fetal descending aorta. All Cesarean sections were performed before the onset of active labor. After removal of macroscopic clot and attached decidua, chorionic villous tissue was separated from primary stem vessels and snap-frozen in the liquid nitrogen, and then stored at -70 °C. First trimester chorionic villi were obtained from five pregnant women at the time of legal induced abortion performed at eighth to tenth week of gestation, and processed as described above. The whole procedure of sampling was completed within 15 min from recovery of the tissue.

Umbilical venous blood was obtained at the time of elective Cesarean sections from five pregnant women with PIH (gestational age; 35.8 ± 3.6 weeks), including the above mentioned four women, and from 12 normotensive pregnant women who had undergone elective repeat Cesarean section (gestational age; 38.7 ± 1.3 weeks). Informed consent was obtained from each patient. Clinical data from patients with PIH are listed in table 1.

Blood sampling and extraction: 10 mL of umbilical cord blood was immediately mixed with 1 mL of saline solution containing 10 mg of EDTA and 10,000 KIU of aprotinin, and was centrifuged at 2,000 x g at 4 °C for 15 min. Plasma samples were stored at -20 °C until assayed. ET-1-LI was extracted from plasma samples using polystyrene beads coated with monoclonal anti-ET-1 antibody as described previously [24].

Radioimmunoassay for ET-1: Radioimmunoassay (RIA) for ET-1 was performed using an anti-ET-1 monoclonal antibody as described previously [8, 24, 36]. The cross-reactivities of the antibody (KY-ET-1-IV) for ET-2, ET-3 and bigET-1 were 80%, 20% and 80% on a molar basis, respectively. The sensitivity of this RIA was 0.2 pg/tube, and the 50% inhibitory concentration was 2 pg/tube. Intra- and interassay coefficients of variation were 7.2% (n = 20) and 11.6% (n = 20), respectively.

Tissue extraction: Chorionic villous tissues were weighed and homogenized using a Polytron homogenizer for 1 min (setting 7) in 10 volumes of ice-cold 1N acetic acid containing 0.1% Triton X-100 as previously described [30]. Homogenates were then boiled at 95 °C for 7 min to inactivate proteases. After centrifugation at 30,000 x g for 30 min at 4 °C, the supernatant was stored at -20 °C until assay. ET-1-LI in the supernatant was subjected to RIA as described above without further extraction.

Gel permeation chromatography (GPC): Tissue homogenates were extracted using Sep-pak C-18 columns (Waters Assoc., Milford, M.A.) and were applied to a Sephadex G-50 column at 4 °C as described [24]. The column was calibrated with blue dextran, human bigET-1, and ET-1. ET-1-LI in each fraction was measured by RIA.

Reverse-phase high performance liquid chromatography (RP-HPLC): Peak GPC fractions were re-extracted using Sep-pak C-18 columns and applied to an octadecyl-silica column (CLC-ODS 0.6 x 15 cm, Shimadzu, Kyoto, Japan) at 30 °C and eluted at a flow rate of 1 mL/min with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. One mL fractions were collected and ET-1-LI in each fraction was measured by RIA.

Ligand binding assay: Chorionic villous tissues were homogenized using a Polytron homogenizer (setting 7) three times for 15 sec each time in

10 volumes of ice-cold homogenization buffer containing 50 mM Tris (pH7.5), 0.1 mM EGTA, 154 mM NaCl, 10 μg/mL pepstatin, 10 μg/mL leupeptin, 100 μg/mL phenylmethylsulphonylfluoride (PMSF), and 10 μg/mL aprotinin (6,400 KIU/mg tissue). The homogenate was centrifuged at 1,000 x g for 30 min at 4 °C, and the supernatant was centrifuged at 40,000 x g for 30 min at 4 °C. The resulting pellet was washed twice and the pellet of the final centrifugation was then resuspended in the same buffer at a final protein concentration of 0.5-1.0 mg/mL and stored at -70 °C until assay. Protein concentration was measured by the method of Bradford et al. [4].

Aliquots of membrane suspension of chorionic villous tissue were incubated in receptor assay buffer (25 mM Hepes in Hanks' balanced salt solution, pH 7.0) containing 0.2% BSA (Sigma, St. Louis, MO), 10 μg/mL pepstatin, 10 μg/mL leupeptin, 100 μg/mL PMSF and 10 μg/mL aprotinin with varying amounts (2 - 128 pM) of [^{125}I]ET-1 or [^{125}I]ET-3 (2,000 Ci/mmol, Amersham, Buckinghamshire, U.K.) for 90 min at 25 °C (total volume 250 μL). The reaction was stopped by chilling in an ice-cold water bath. Membrane-bound radioactivity was separated from free ligands by rapid filtration through Whatman GF/C glass fiber filters and the radioactivity associated with the filters was measured with a gamma counter at 60% efficiency. Specific binding was defined as the difference between the total binding and the nonspecific binding determined in the presence of 100 nM unlabeled ET-1 or ET-3.

Total RNA extraction and Northern blot analysis: Total RNA was extracted from chorionic villous tissue by the guanidinium thiocyanate/CsCl method [27]. Total RNA (20 μg) was fractionated on a 0.66 M formaldehyde-1% agarose gel, transferred on to a nylon membrane and hybridized with ^{32}P-labeled human ET-AR and ET-BR cDNA fragments as previously described [10, 21].

Statistical analysis: Data are expressed as the means ± SD. Statistical comparisons between the groups were carried out by Student's *t* test. P values less than 0.05 were regarded as significant.

Results

ET-1-LI in the umbilical venous plasma of newborns from PIH women was 38.3 ± 10.4 pg/mL (n = 5), significantly higher than that of newborns from normotensive pregnant women (26.3 ± 5.2 pg/mL, n = 12, $P < 0.05$).

ET-1-LI in the homogenate of chorionic villous tissue was measured by RIA. The serial dilution curve of extracts of chorionic villous tissue paralleled to the standard curve of ET-1 (data not shown). Figure 1 shows a comparison of ET-1-LI levels in the homogenates of first trimester chorionic villous tissue, and third trimester chorionic villous tissues obtained from normotensive and PIH women. ET-1-LI level in the homogenates of chorionic villous tissue in the first trimester was 210 ± 68 pg/g wet weight (n = 5). A strikingly high level of ET-1-LI was observed in homogenates of chorionic villous tissues from normal pregnant women at term (2,450 ± 940 pg/g wet weight, n = 4) and in those from women with PIH (2,850 ± 740 pg/g wet weight, n = 4), but these values were not significantly different from each other.

Figure 2 shows a typical GPC profile and RP-HPLC pattern of ET-1-LI in homogenates of chorionic villous tissues from women complicated with PIH. As shown in Figure 2(A), ET-1-LI in the chorionic villous tissue from PIH-complicated pregnancies was predominantly eluted at the same position as synthetic ET-1 (n = 2). ET-1-LI in this peak fraction was further analyzed by RP-HPLC, in which ET-1-LI was mainly eluted at the same position as synthetic ET-1 (Figure 2(B)). Several minor peaks were also observed, all of which were at positions different from any synthetic ETs. Exactly equivalent patterns were observed in GPC and RP-HPLC profiles of ET-1-LI in homogenates of chorionic villous tissue of normal term placentae (n = 4, data not shown).

Ligand binding analyses for [^{125}I]ET-1 and [^{125}I]ET-3 were carried out using membrane fractions of placental chorionic villous tissue. Binding of [^{125}I]ET-1 and [^{125}I]ET-3 to the placental membrane fraction reached an apparent equilibrium after 90 min incubation at 25 °C (data not shown). Figure 3 illustrates typical saturation binding isotherms and Scatchard plots of [^{125}I]ET-1 and [^{125}I]ET-3 to the membrane fraction of chorionic villous tissue obtained from normotensive pregnancy. Table 2 summarizes the apparent Kd and Bmax values for [^{125}I]ET-1 and [^{125}I]ET-3. In the chorionic villous tissue of term placentae from normotensive women, a single class of high affinity ET-receptor sites with apparent Kd of 23.6 ± 11.1 pM and Bmax value of 388 ± 238 fmol/mg protein for [^{125}I]ET-1 were identified (n = 5). The Kd and Bmax values for [^{125}I]ET-3 were 13.9 ± 3.8 pM and 176 ± 78 fmol/mg protein (n = 5), respectively. In the chorionic villous tissue of placentae from women complicated with PIH, Kd values for both [^{125}I]ET-1 and [^{125}I]ET-3 were almost identical to those in normotensive pregnant women. Bmax values for [^{125}I]ET-1 and [^{125}I]ET-3 (263 ± 51 fmol/mg protein and 127 ± 21 fmol/mg protein, respectively) were slightly lower than those in the chorionic villous tissue of normotensive pregnant women. However, the differences were not statistically significant. Although the ET concentration in the first trimester chorionic villous tissue was extremely low, the Bmax value in this tissue was comparable to that in the chorionic villous tissue of term placentae. The ratio of Bmax value for [^{125}I]ET-3 to that for [^{125}I]ET-1 were similar in both normal and PIH placentae (Table 2).

As shown in Figure 4, Northern blot analysis using human ET-AR and ET-BR cDNA probes detected substantial levels of both ET-AR and ET-BR mRNAs in chorionic villous tissues of term placenta and in the first trimester chorionic villous tissue; a single 4.3 kb band and two bands of 4.3 kb and 1.7 kb were detected, respectively.

Discussion

In the present study, we observed a high concentration of ET-1 (approximately 1 pmol/g wet weight) in the chorionic villous tissue of human placenta at term. ET-1 at this concentration has been reported to elicit substantial effects on various cell functions including proliferation of placental fibroblasts and contraction of cotyledonary vessels [7, 19, 34, 35]. Thus, it is probable that ET-1 in this tissue is of some physiological significance. Other ET isopeptides (ET-2 or ET-3) were not detected in term placentae in the present study, although the expression of ET-3 mRNA was reported in this tissue [22].

We and others have reported previously that plasma ET-1-LI levels in pregnant women with PIH are elevated compared with those in the normotensive pregnant women [5, 11, 13, 17, 20, 28, 32], although several investigators have reported contradictory results [3, 16]. In the present study, ET-1-LI levels in the umbilical venous plasma of newborns from PIH women were also higher than those in newborns from normotensive pregnant women. These findings suggest that the elevated levels of plasma ET-1-LI observed on both sides of placental circulation in PIH may be a consequence of enhanced ET-1 production in placental tissue, which may be related to the central events of this disorder such as reduced uteroplacental blood flow and increased placental vascular resistance [14, 33].

In the present study, however, ET-1-LI levels in placentae from PIH women were not different from those in the placentae from normotensive pregnant women. Furthermore, the molecular form of ET in the placentae of both of these groups were almost identical. These results suggest that the increases in ET-1-LI observed in the plasma of both mother and fetus during pregnancy complicated with PIH does not originate from the placenta, but may originate from other vascular beds or reflect altered

ET metabolism. Similar findings were reported by Benigni et al. [3], but the reported ET concentration and the molecular form of ET were both quite different from our data, probably due to differences in extraction procedures between this previous and the present study.

High affinity receptor sites for [^{125}I]ET-1 and [^{125}I]ET-3 were detected in membrane preparations from term placentae and first trimester chorionic tissues. The calculated Bmax value for [^{125}I]ET-3 was approximately half of that for [^{125}I]ET-1 in both term placentae and first trimester chorionic tissues. To date at least two types of ET receptors, ET-AR and ET-BR, have been reported [10,21]. The affinity of ET-3 for human ET-AR when expressed in transfected cells is two orders of magnitude lower than that of ET-1 [10], and the affinities of ET-isopeptides for ET-BR are the same [21]. Thus, the results of the present binding assay indicate that ET-AR and ET-BR are expressed almost equally in term placentae and in first trimester chorionic tissues, provided that ET-AR and ET-BR are the only ET receptors present. Northern blot analysis confirmed that ET-AR and ET-BR genes are expressed in human placentae at term. ET-AR and ET-BR have been reported to be expressed in vascular smooth muscle cells and vascular endothelial cells, respectively [10, 21]. Placenta has abundant vascular tissue, which may become a target of placental endothelin. In addition, in our preliminary experiment, the intracellular calcium ion concentration of human placental trophoblast cells in primary culture was increased equally by addition of either ET-1 or ET-3, suggesting the presence of ET-BR in these cells (Yano J & Sagawa N, unpublished observation). Thus, it is plausible that the high concentrations of ET-1 observed in the placenta may bind to the abundant ET receptors there and modulate various placental functions.

In the placentae from women with PIH, Bmax values for [^{125}I]ET-1 and [^{125}I]ET-3, and their ratio (Bmax <ET-3>/Bmax <ET-1> ratio) were

essentially the same as those in normal placentae, suggesting that the ET system in the placenta is not altered in PIH.

In conclusion, an extremely high concentration of ET-1 and abundant ET-AR and ET-BR were identified in normal human placentae at term, suggesting a possible biological role of ET-1 in this tissue. However, concentrations of ET-1 and ET receptors in placentae from PIH women were not different from those in the normal placentae. Thus, the elevated levels of plasma ET-1-LI observed in both maternal and fetal circulation during pregnancies complicated by PIH cannot be related to changes in the ET system in the placenta. Further investigation of the effects of ET on trophoblast function or on placental vascular contraction (tone) may provide more information on the physiological and pathophysiological role of the ET system in the human placenta.

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Table 1. Clinical features of PIH women.

Case #	Gestational age (weeks)	Parity	Blood pressure (mmHg)	Urine protein (g/day)	Birth weight (g)	Deviation from mean birth weight	Apgar score (1 min)
case 1	31	0	186/116	1	750	-3.1 SD	1
case 2	36	0	219/139	1	1640	-1.8 SD	7
case 3	35	0	200/110	3	1920	-1.2 SD	6
case 4	41	0	144/102	10	2350	-2.4 SD	8
case 5*	36	0	169/102	3	2166	-1.1 SD	8

*; Placenta was not analyzed in this case.

Table 2. Bmax and Kd values of [^{125}I]ET-1 and [^{125}I]ET-3 binding to the membrane fractions of chorionic villous tissues of first and third trimester of pregnancy.

Tissue	Bmax(ET-1) (fmol/mg prot.)	Kd(ET-1) (pM)	Bmax(ET-3) (fmol/mg prot.)	Kd(ET-3) (pM)	Bmax(ET-3)/ Bmax(ET-1) ratio
First trimester					
Normal (n=5)	290 ± 171	18.6 ± 2.5	169 ± 78	15.1 ± 4.3	0.62 ± 0.12
Third trimester					
Normal (n=5)	388 ± 238	23.6 ± 11.1	176 ± 78	13.9 ± 3.8	0.50 ± 0.17
PIH (n=4)	263 ± 51	22.6 ± 7.6	127 ± 21	14.2 ± 3.3	0.48 ± 0.03

Values are the means ± SD.

Legends for Figures

Figure 1. Levels of ET-1-LI in the homogenates of first trimester chorionic villous tissue and chorionic villous tissues from both normotensive and PIH women in the third trimester.

Values are the means ± SD.

Figure 2. Typical GPC profile (A) and RP-HPLC pattern (B) of ET-1-LI in the homogenate of chorionic villous tissue of term placentae.

The extract from chorionic villous tissue of PIH-complicated pregnancy was subjected to GPC and RP-HPLC as described in Materials and Methods. Vo, void volume; Vt, total volume.

Figure 3. Typical saturation isotherms of [^{125}I]ET-1 and [^{125}I]ET-3 binding to the membrane fraction of chorionic villous tissue of normal pregnancy at term.

Membranes (6-9 μg protein) were incubated with various concentrations of [^{125}I]ET-1 or [^{125}I]ET-3 for 90 min at 25 °C. Nonspecific binding (NSB) was determined by incubation in the presence of 100 nM unlabeled ET-1 or ET-3. Scatchard plots of the binding data are shown in the inset. Closed circles; total [^{125}I]ET-1 binding, Closed squares; total [^{125}I]ET-3 binding, Open circles; NSB of [^{125}I]ET-1, Open squares; NSB of [^{125}I]ET-3.

Figure 4. Northern blot analysis of total RNA from first trimester chorionic tissue and chorionic villous tissues from both normotensive and PIH women in the third trimester.

Total RNA (20 μg) from chorionic villous tissues were analyzed as described in Materials and Methods.

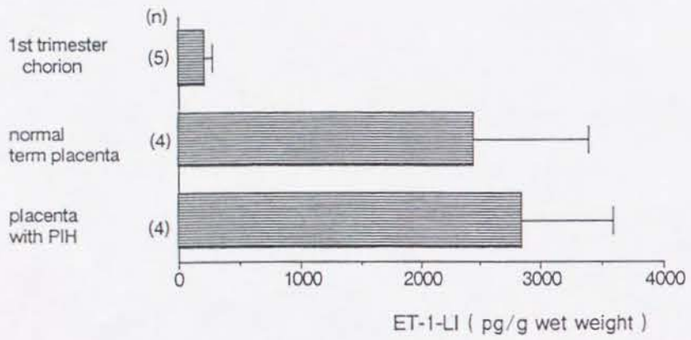

Figure 1

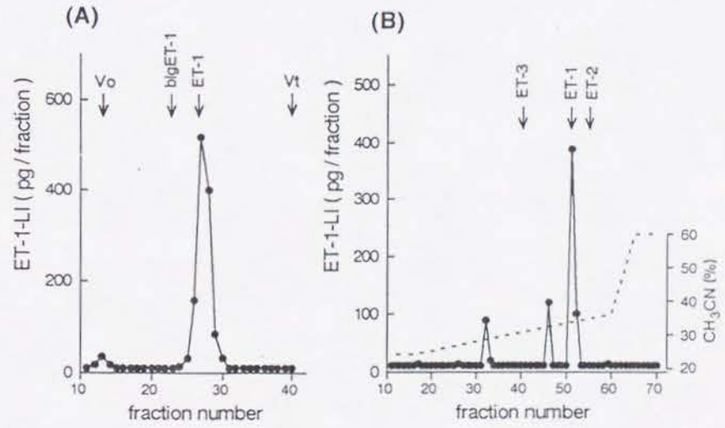

Figure 2

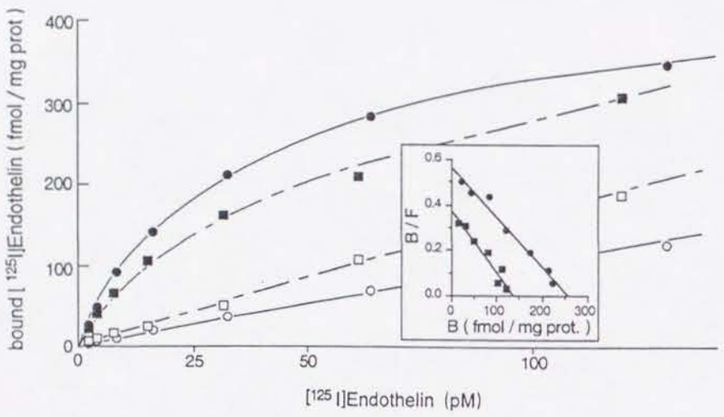

figure 3

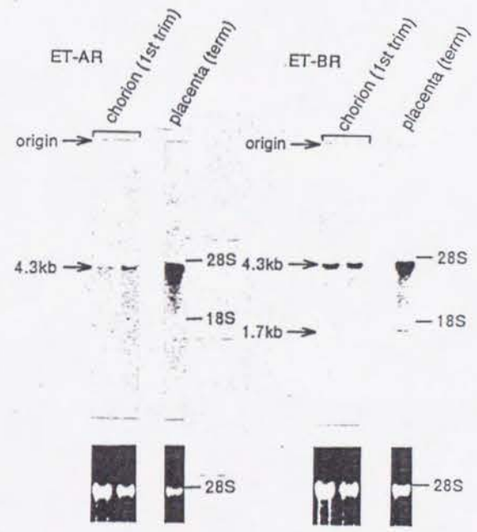

Figure 4

Summary

To elucidate the possible role of endothelin (ET) in the pathophysiology of pregnancy-induced hypertension (PIH), the concentration of ET and its receptors in the chorionic villous tissue of placenta as well as ET concentration in the umbilical venous plasma from normal and PIH-complicated pregnancy were determined.

Placentae were obtained from four PIH-complicated women who had undergone Cesarean section due to fetal distress, and five normal pregnant women who had undergone repeat Cesarean sections. Chorionic tissue in the first trimester was obtained from five pregnant women at the time of legal induced abortion performed at eighth to tenth week of gestation. Umbilical venous blood was obtained from five newborns delivered from women with PIH by emergency Cesarean sections due to fetal distress, and from twelve newborns delivered from normal pregnant women by elective Cesarean section before labor onset at term. ET-1-like immunoreactivity (ET-1-LI) in the tissue homogenate and plasma samples were measured by a sensitive RIA as described previously [22].

High levels of ET-1-LI were detected in the chorionic villous tissue of term placenta (2,450 ± 940 pg/g wet weight, mean ± SD, n = 4) (Figure 1). Further analysis using gel permeation chromatography (GPC) and reverse-phase high performance liquid chromatography (RP-HPLC) revealed that the main constituent of ET-1-LI in this tissue was ET-1 (Figure 2). Scatchard analysis of [^{125}I]ET saturation binding in the chorionic villous tissue of term placenta showed linear plots indicating a single class of binding sites with apparent dissociation constant (Kd) of 23.6 ± 11.1 pM and 13.9 ± 3.8 pM, and Bmax values of 388 ± 238 fmol/mg protein and 176 ± 78 fmol/mg protein, for [^{125}I]ET-1 and [^{125}I]ET-3 (n=5), respectively (table II). This was supported by the Northern blot analysis

which showed that both ET-A and ET-B receptor genes are expressed in this tissue (Figure 4).

Comparable ET receptor sites and remarkably lower ET-1-LI levels were observed in the first trimester chorion (Figure 1, table II).

In the chorionic villous tissue of placenta from PIH-complicated women, both ET-1-LI and ET binding profiles were not different from those in the normal pregnancy (Figure 1, table II), whereas ET-1-LI in the umbilical venous plasma from these hypertensive patients (38.3 ± 10.4 pg/mL, n = 5) were significantly ($P<0.05$) elevated when compared with those in the normal newborns (26.3 ± 5.2 pg/mL, n = 12).

These results suggest that ET system may not be directly involved in the development of placental dysfunction occurring in PIH.

In conclusion, an extremely high concentration of ET-1 and abundant ET-AR and ET-BR were identified in the human normal placenta at term. However, the elevated levels of plasma ET-1-LI observed in both maternal and fetal circulation in pregnancy complicated with PIH could not be related with the changes in the ET production or concentrations of ET receptors in the placenta. Further study is mandatory to elucidate the role of ET system in the pathophysiology of placenta in PIH.

Curriculum Vitae

MASAAKI HASEGAWA, M.D. graduated from Kyoto University School of Medicine in 1983. After six years of internship and clinical training on Obstetrics and Gynecology in the Kyoto University Hospital and the Red Cross Hospital at Osaka, he started a research work in the Departments of Obstetrics and Gynecology and Internal Medicine, Kyoto University Faculty of Medicine. The major theme of his research is the role of endothelin in the human pregnancy.